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## A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically

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### ABSTRACT

Apple replant disease (ARD) has been reported from all major fruit-growing regions of the world, and is often caused by a consortium of biological agents. The aim of this study was to investigate the etiology of ARD in South Africa in six orchard soils, using a multiphasic approach under glasshouse conditions. This approach first involved determining the ARD status of the soils by monitoring apple seedling growth responses in non-treated soil versus growth in pasteurized soil, as well as in 15% non-treated soil that was diluted into pasteurized soil. Subsequently, the potential for specific organisms to function as causal agents of ARD was investigated using (i) biocide applications, (ii) quantitative real-time PCR (qPCR) analyses of ARD 'marker' microbes (*Pythium irregulare*, *Pythium sylvaticum*, *Pythium ultimum*, *Pythium vexans*, *Rhizoctonia solani* AG-5 and the genera *Cylindrocarpon* and *Phytophthora*), (iii) nematode analyses, (iv) isolation of actinomycetes and (v) pathogenicity testing of actinomycetes individually, and when co-inoculated with *P. irregulare* or *Cylindrocarpon macrodidymum*. The analyses showed that the soils could be grouped into low, moderate and severe ARD soils, with each group containing two soils. Several lines of evidence suggested that actinomycetes are not involved in ARD in South Africa. Multiple biological agents were determined to contribute to ARD including (i) oomycetes (*Phytophthora* and *Pythium*) that are important based upon their widespread occurrence, and the fact that metalaxyl application improved seedling growth in four soils (ii) the genus *Cylindrocarpon* that was also widespread, and for which a synergistic interaction with *P. irregulare* was demonstrated and (iii) occasionally parasitic nematodes, mainly *Pratylenchus penetrans*, *Pratylenchus scribneri* and *Pratylenchus delattrei*, since fenamiphos application improved seedling growth in two orchards. qPCR analyses of the ARD marker microbes showed that *R. solani* AG-5 is absent from South African orchards, and that *P. ultimum* is widespread, even though the latter species could not be detected in previous isolation studies. The other marker microbes were also widespread, with the exception of *P. sylvaticum*. qPCR quantification of the marker microbes could not be correlated with the severity of ARD in any manner. qPCR analyses did, however, show that possible root pruning pathogens such as *P. irregulare*, *P. sylvaticum* and *P. ultimum* had much lower DNA concentrations in seedling roots than *P. vexans* and the genera *Cylindrocarpon* and *Phytophthora*.

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### 1. Introduction

Young apple trees that are planted on sites that were previously cultivated with apple or closely related species often exhibit poor growth. It is generally assumed that this poor growth is most severe on sites that were planted to apple for extended periods of time

(Mai and Abawi, 1981). However, symptomatic trees have also been noticed after apples had been grown in soil for only one year (Savory, 1966). Microbial communities consistent with replant disease have also been documented to develop within three years of orchard establishment (Mazzola, 1999). The phenomenon of poor growth on replanted apple soils is characterized by its persistence in soil, and its lack of spread through replant sites. Therefore, the effect is most evident when trees are replanted into the old tree rows (Hoestra, 1968; Jensen and Buszard, 1988; Mazzola, 1998b; Rumberger et al., 2004; Leinfelder, 2005).

Symptoms associated with poor tree growth are neither distinctive nor always dramatic (Jackson, 1979; Sewell, 1981). The

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most obvious aboveground symptom is the uneven growth of young trees within an orchard. However, when severe disease pressure is encountered, poor growth may be exhibited by the majority of trees in the orchard and death of young trees may occur (Traquiar, 1984). Below-ground symptoms include small root systems that have a significant reduction in lateral root development and functional root hairs (Savory, 1966; Hoestra, 1968; Caruso et al., 1989). Trees that are affected often also begin cropping fruit 2 to 3 years later than unaffected trees and fail to attain comparable yields (Mazzola, 1998a).

Apple replant disease (ARD) or soil sickness, refers to situations where poor growth of trees is caused by a biological component (Savory, 1966; Mai and Abawi, 1981; Traquiar, 1984; Gilles and Bal, 1988). In general, demonstration of an ARD-inducing soil requires that (i) soil pasteurization or fumigation improves tree growth relative to non-fumigated soil (Hoestra, 1968; Covey et al., 1979; Mai and Abawi, 1981; Jaffee et al., 1982a; Slykhuus and Li, 1985) and/or (ii) dilution of the soil into pasteurized soil with as low as 10% of the original field soil can still cause stunting of trees (Hoestra, 1968; Jaffee et al., 1982a).

Numerous biological agents that vary across orchards have been implicated in ARD, but among these only a few pathogenic nematodes, fungi and oomycetes species have been reported world-wide. *Pratylenchus penetrans* Cobb is considered to be the primary nematode species involved in ARD (Mai et al., 1957; Jaffee et al., 1982b; Merwin and Stiles, 1989; Utkhede et al., 1992a; Dullahide et al., 1994; Van Schoor et al., 2009). Within the fungal genus *Cylindrocarpon*, which in general is considered to have low virulence, *Cylindrocarpon destructans* (Zinns.) Scholten and *Cylindrocarpon lucidum* Booth have been reported as the dominant and pathogenic species associated with ARD in a few regions of the world (Jaffee et al., 1982a; Braun, 1991, 1995; Dullahide et al., 1994; Mazzola, 1998a; Manici et al., 2003). In South Africa, four pathogenic *Cylindrocarpon* species have been reported including *Cylindrocarpon macrodidymum* Schroers, Halleen & Crous, *C. destructans*, *C. liriodendri* Halleen, Schroers, Groenewald, Rego, Oliveira & Crous and *C. pauciseptatum* Schroers, Zerjav, Munda, Halleen & Crous, with *C. macrodidymum* being the most wide-spread (Tewoldemedhin et al., 2011a). Several *Rhizoctonia* species have been associated with ARD, but only the multinucleate *R. solani* Kühn AG-5 and AG-6 were shown to be highly virulent, whereas a few of the binucleate anastomosis groups have exhibited low virulence toward apple (Mazzola, 1997; Manici et al., 2003). Although *Fusarium* (mainly *Fusarium oxysporum* Schlechtend.) is frequently associated with ARD, its role as a pathogen of apple is controversial. Most studies were unable to demonstrate that *Fusarium* isolates are pathogenic (Merwin and Stiles, 1989; Dullahide et al., 1994; Mazzola, 1998a; Manici et al., 2003; Tewoldemedhin et al., 2011b). However, *F. tricinctum* (Corda) Sacc. and some isolates of *F. solani* (Mart.) Sacc. and *F. avenaceum* (Fr.) Sacc. have been shown to be pathogenic, with the latter two species having low virulence (Dullahide et al., 1994; Manici et al., 2003; Tewoldemedhin et al., 2011b). In most countries several pathogenic *Phytophthora* species have been identified, with *P. cactorum* (Leb. and Cohn) Schröeter being the dominant species (Sewell, 1981; Matheron et al., 1988; Utkhede et al., 1992a; Mazzola, 1998a; Tewoldemedhin et al., 2011b). In contrast, not all *Pythium* species are pathogenic with some even promoting the growth of apple seedlings (Mazzola et al., 2002). The most virulent species that have been identified and have also been frequently associated with ARD include *P. intermedium* de Bary, *P. irregulare* Buisman, *P. sylvaticum* Campbell & Hendrix, *P. ultimum* Trow and *P. vexans* de Bary (Sewell, 1981; Jaffee et al., 1982a; Dullahide et al., 1994; Mazzola, 1998a; Mazzola et al., 2002; Tewoldemedhin et al., 2011b).

Only a few studies have focused on the role of prokaryotes in ARD, and in general their role is still controversial (Savory, 1966;

Hoestra, 1968; Mazzola, 1998a; Dullahide et al., 1994). Although several bacterial genera and species have been associated and suggested as being involved in ARD, only isolates of *Bacillus subtilis* have been shown to limit plant growth (Catska et al., 1982; Utkhede et al., 1992b). However, the inoculum concentrations used in the latter study were inordinately high ( $8.8 \times 10^9$  to  $1.2 \times 10^{11}$  colony forming units/500 cm<sup>3</sup> soil), and most bacteria are likely to limit plant growth and development at these densities (Klement et al., 1990; Schaad et al., 2001).

Evidence for the involvement of actinomycetes in ARD is circumstantial (Savory, 1967; Hoestra, 1968; Westcott et al., 1986). Westcott et al. (1986, 1987) found through microscopic analyses that the extent of colonization of apple root epidermal tissue by actinomycete-like organisms was positively correlated with ARD severity, whereas roots in steamed soil were not infected by actinomycetes. More recently, Zhao et al. (2009) showed that certain actinomycetes, specifically *Streptomyces* species, which were isolated from brassicaceous seed meal amended apple soils, could alleviate *R. solani* AG-5 root infections but that some of the isolates by themselves caused leaf necrosis and a reduction in root biomass in young apple seedlings. The latter response was eliminated when older seedlings were employed in these assays. On the other hand, some of the *Streptomyces* isolates were able to suppress *R. solani* AG-5 infections and thus stimulate seedling growth (Cohen and Mazzola, 2006; Zhao et al., 2009).

A diverse range of approaches have been taken to elucidate the complex etiology of ARD. Most studies have used isolation studies along with pathogenicity testing (Jaffee et al., 1982a; Braun, 1991, 1995; Dullahide et al., 1994; Mazzola, 1998a; Manici et al., 2003). Additionally, the application of biocides has been used to suppress certain components of the pathogen complex in order to deduce the importance of specific groups (Mai and Abawi, 1978; Slykhuus and Li, 1985; Dullahide et al., 1994; Mazzola, 1998a). More recently, polymerase chain reaction (PCR) based techniques including DNA fingerprinting with denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphisms (T-RFLP), as well as sequencing of root infected clone libraries have also been used to characterize microbial populations in apple orchard soils (Rumberger et al., 2004, 2007; Yao et al., 2006; St. Laurent et al., 2008). Although these molecular techniques yield a vast amount of data on microbial community structure, the studies were unable to identify specific ARD causal agents nor specific genera or species to serve as predictors of potential growth reductions.

Real-time PCR or quantitative PCR (qPCR) is a powerful tool for detection and quantification of microbial genera or species of interest from soil and plant material (Schna et al., 2004; Lievens et al., 2005; Paulitz and Schroeder, 2005; Kernaghan et al., 2007; Ophel-Keller et al., 2008). Although several studies have conducted qPCR analyses on some of the known agents associated with ARD, they were on crops other than apple (Paulitz and Schroeder, 2005; Schroeder et al., 2006; Kernaghan et al., 2007, 2008; Hoagland et al., 2008; Schna et al., 2008). With regard to replant diseases in general, Bent et al. (2009) have recently shown that the amount of *P. ultimum* and *Sellaphora* DNA in roots from peach seedlings grown in replant soil was negatively associated with plant biomass, whereas *P. vexans* and *Aplanochytrium* did not show a significant association with plant biomass.

In South Africa, some information has been obtained on the biological agents involved in ARD. Van Schoor et al. (2009) showed that ARD in South Africa is primarily caused by a biological phenomenon. They reported that the genera *Pythium*, *Cylindrocarpon* and *Fusarium* were consistently isolated from replant soils, whereas *Rhizoctonia* and *Pratylenchus* were inconsistently associated with ARD soils. However, no species level identifications or pathogenicity studies were conducted (Van Schoor et al., 2009).

Recent studies have described the species of *Cylindrocarpon*, *Fusarium*, *Pythium*, *Phytophthora* and *Rhizoctonia* resident in six apple orchard soils using isolation techniques, and characterized the pathogenicity of several species from these genera toward apple (Tewoldemedhin et al., 2011a,b).

In order to increase our understanding of ARD it is best to use a multiphasic approach that includes traditional isolation and pathogenicity studies, along with the use of biocide applications and molecular methods. In the current study, this approach was investigated and incorporated results from previous isolation and pathogenicity studies (Tewoldemedhin et al., 2011a,b). The study specifically aimed to investigate in six apple orchards (1) the severity of ARD and the biological agents involved as revealed through biocide applications and qPCR, (2) if multiple agents (*Pythium* and *Cylindrocarpon*) can cause greater plant damage than when acting independently and if these agents can act synergistically with actinomycetes, (3) whether there is a correlation between pathogen DNA concentrations in roots and seedling growth inhibition and (4) whether ARD agents in South Africa are similar to those in other regions of the world and if actinomycetes play a role.

## 2. Materials and methods

### 2.1. Soil sampling and analyses

Soil samples were collected from six orchards that were cultivated to apple (*Malus domestica* Borkh.) for more than 15 years. The orchards were situated in the Grabouw (hereafter denoted Orchards O1, O2 and O3) and Ceres (Orchard O4, O5 and O6) areas in South Africa (Tewoldemedhin et al., 2011a,b). Except for Orchard O4, all soil samples were collected from within the old tree row. In Orchard O4, the old tree rows had been fumigated with methyl bromide at the time of sampling, and therefore soil samples were collected between the old tree rows. In each orchard five independent samples were collected at a depth of 5–25 cm, which were mixed thoroughly to represent each orchard in the glasshouse trial. Several chemical and physical characteristics of the soils were analyzed (Table 1). The analyses were conducted by BemLab (edms) Bpk (Somerset West, South Africa) according to published protocols (Jones, 2001).

### 2.2. Biocide and replant growth inhibition glasshouse trial

The six orchard soils each received five different treatments, which included (i) pasteurization, (ii) dilution of each orchard soil into the corresponding pasteurized orchard soil at a 15% rate, and the application of (iii) metalaxyl, (iv) difenconazole or (v) fenamiphos.

Soils were pasteurized for three hours at 82 °C for two consecutive days, three days prior to planting. Metalaxyl, difenconazole and fenamiphos were applied as an atomized mist (Mazzola et al., 1992) with constant mixing of the soil during application to obtain uniform distribution of the chemicals. NemaCur 3 (a.i. fenamiphos; Bayer, Isando, South Africa) was applied at a final rate of 0.146 µg a.i. g<sup>-1</sup> to suppress nematodes. Ridomil 2E (a.i. metalaxyl; Syngenta SA (Pty) Ltd, Halfway House, South Africa) was applied at a final rate of 0.0052 µg a.i. g<sup>-1</sup> to suppress the oomycetes *Phytophthora* and *Pythium*. Score 250 E (a.i. difenconazole; Syngenta SA (Pty) Ltd, Halfway House, South Africa) was applied at a final rate of 0.0083 µg a.i. g<sup>-1</sup> to suppress a wide range of fungi including *Cylindrocarpon*, *Fusarium* and *Rhizoctonia*.

Four-week-old apple seedlings (Golden Delicious) were produced for use in the glasshouse trials. Apple seeds treated with Captan (Dow AgroSciences Southern Africa (Pty) Ltd, Silverton, South Africa) were stratified for three months. The stratified seeds were then placed, after another Captan treatment, in perlite for two weeks in a growth chamber at ±25 °C. Germinating seeds were transplanted into seedling trays containing a sterile sand and bark (2:1 v/v) growth medium. Seedlings were grown in a glasshouse at 20–25 °C for 4 weeks, prior to use in the glasshouse trials. Treated and non-treated soils were dispensed into one litre plastic bags and three four-week-old seedlings were planted into each bag. The experiment was conducted using a randomized complete block design with each treatment containing six replicates, and a replicate consisting of one bag containing three seedlings. Seedlings were grown for three months in a glasshouse with temperatures ranging from 20 to 30 °C, and the trial was conducted twice.

After three months, the increase in seedling weight and height was determined as previously described (Tewoldemedhin et al., 2011a). The ARD severity of the soils was determined for each soil by calculating the mean weight (g) difference of seedlings grown in pasturized soil and in non-treated soil for each specific orchard. Statistical analyses conducted on the trial results for this section are described in Section 2.7. Roots of seedlings grown in the non-treated soil were partitioned into subsamples for use in several subsequent analyses including (i) actinomycete isolations, (ii) nematode analyses and (iii) qPCR analyses. Roots of seedlings grown in pasteurized soils were used for actinomycete analyses and as negative controls in qPCR analyses.

### 2.3. Isolation and identification of actinomycete genera and genotypes

Roots were washed under running tap water to remove adhering soil, and roots were divided into two groups. The first

**Table 1**

Soil characteristics of old apple orchard soils and their apple replant disease (ARD) status as revealed through the growth response (weight) of apple seedlings grown in orchard soils that were steam pasteurized or left untreated.

Orchard code	Growth response in pasteurized and un-treated soil		Soil characteristics						
	P-NT <sup>a</sup>	ARD test result	Soil texture	pH	Resistance <sup>c</sup>	Mg <sup>d</sup>	Ca <sup>d</sup>	P <sup>e</sup>	C <sup>f</sup>
O1	8.35 c <sup>b</sup>	Low	Clay	6.3	660	1.21	6.27	26	1.74
O4	7.3 c	Low	Loam	6.6	2120	2.05	9.30	49	1.61
O3	16.4 b	Moderate	Loam	6.4	1280	1.44	8.24	78	1.87
O5	15.55 b	Moderate	Loam	5.5	1860	1.52	7.85	44	2.39
O2	18.991 a	Severe	Sand	5.9	850	1.52	6.66	101	2.18
O6	18.554 a	Severe	Sand	6.3	2840	1.65	6.17	173	1.63

<sup>a</sup> Values are the mean weight (g) difference of seedlings grown in pasturized and in non-treated soil within each specific orchard. The values are the means of two independent experiments that each contained six replicates.

<sup>b</sup> Means within each parameter followed by the same letter do not differ significantly ( $P=0.05$ ).

<sup>c</sup> Resistance (ohm).

<sup>d</sup> Magnesium and Calcium (cmol(+)/kg).

<sup>e</sup> Available phosphorus (mg/kg).

<sup>f</sup> Percent organic carbon.

group (group A) was subjected to a three-step surface sterilization procedure including a 60-s wash in 99% ethanol, followed by a 6 min wash in 3.125% NaOCl, a 30 s wash in 99% ethanol and a final rinse in sterile water (Coombs and Franco, 2003). The second group (group B) was only subjected to a 30 s wash in 99% ethanol. Surface-sterilized roots were aseptically sectioned into 1 cm fragments and group A was plated onto Casein-Starch medium (CS) (Wellington and Toth, 1994) and group B onto water agar amended with cycloheximide ( $100 \mu\text{g mL}^{-1}$ ). For each trial, a total of 360 root segments were plated per orchard for seedling replicates grown in non-treated soil, and 360 root segments for seedling replicates grown in pasteurized soil. Culture plates were incubated at  $27^\circ\text{C}$  for up to 4 weeks. Growth on plates that morphologically resembled that of actinomycetes was streaked onto CS medium to obtain single colonies. Purified single colony actinomycete isolates were stored at  $-85^\circ\text{C}$  in a yeast extract malt extract (YEME) (Loria and Davis, 1988) plus glycerol (60%) solution.

DNA was isolated from two-week-old pure cultures of actinomycetes growing on CS medium using a slight modification (Tewoldemedhin et al., 2011a) of the protocol described by Lee and Taylor (1990). The genus and genotypes of isolates within each genus were determined through sequence analysis of the 16S rRNA gene. PCR amplification of the 16S rRNA gene was conducted using primers 8f and 1406r (Amann et al., 1995). PCR reactions and amplification conditions were as previously described (Tewoldemedhin et al., 2011a) except that PCR reactions contained 2 mM  $\text{MgCl}_2$  and amplification conditions consisted of an initial denaturation of 5 min at  $95^\circ\text{C}$ , followed by 35 cycles of 30 s at  $95^\circ\text{C}$ , 1 min at  $54^\circ\text{C}$  and 2 min at  $72^\circ\text{C}$  and a final extension cycle of 7 min at  $72^\circ\text{C}$ . Successful PCR amplification was confirmed through agarose gel analyses. PCR products were purified and sequenced as previously described (Tewoldemedhin et al., 2011a) using primers 8f and 1406r. Single strand sequences of 996-bp to 1270-bp in length were obtained for each isolate, which were used in BLAST searches on GenBank to identify the isolates to the genus level. Isolates that belonged to the genus *Streptomyces* were further grouped into genotypes, based on their similarity to specific GenBank sequences.

#### 2.4. Nematode isolations and identifications

Nematode analyses were conducted from soil and seedling root samples from each of the six non-treated orchard soils. Analyses were conducted by Nemlab Diagnostic Services (Durbanville, South Africa) using the centrifugal sugar flotation method (Jenkins, 1964). A subset of the nematodes was identified to the species level by the Nematology unit of the Biosystematic division of the Agricultural Research Council (Pretoria, South Africa).

#### 2.5. Quantitative real-time PCR analyses of replant fungal and oomycete pathogens

The roots of apple seedlings grown in each of the six non-treated and pasteurized orchard soils were stored at  $-85^\circ\text{C}$  at the end of the glasshouse trial for subsequent DNA extractions and qPCR analyses. A bulk root sample was obtained for each replicate bag by excising an equal amount of root tissue from each of the three apple seedlings, yielding six samples per non-treated and pasteurized orchard soil. Only roots from the second glasshouse trial were used in qPCR analyses, since root DNA from the first trial were degraded due to freezer problems. Frozen root samples were ground to a fine powder using liquid nitrogen and a IKA® A-11 Analytical mill (IKA®-Werke GmbH & Co., Staufen, Germany). DNA was extracted as previously described (Tewoldemedhin et al., 2011a).

All qPCR analyses were conducted using either SYBR® Green or TaqMan chemistry and SensiMix™ dT (Quantace Ltd., London, UK).

Amplifications were conducted in a Rotor-Gene™ 6000 real-time rotary analyzer (Qiagen Inc., Valencia, CA, USA). Each set of qPCR reactions included two replications of each DNA sample, a non template (water) control, a negative control (root DNA obtained from roots grown in pasteurized soil) and two standard curve control (calibrators) samples for each target species. Standard curves were constructed for all the investigated genera and species as previously described (Spies, 2010; Tewoldemedhin et al., 2011a,b). *Cylindrocarpon* DNA concentrations in seedling roots were estimated using two standard curves that consisted of two *Cylindrocarpon* isolates (STE-U6615 *C. destructans* and STE-U6627 *C. liriodendri*) that were isolated from ARD soil in a previous study, and which are available from the Stellenbosch University culture collection (Tewoldemedhin et al., 2011a). These two species are known to yield the highest (*C. destructans*) and lowest (*C. liriodendri*) DNA concentrations (Tewoldemedhin et al., 2011a). qPCR data were analyzed using Rotor-Gene 6000 Series Software 1.7.

Published primer pairs, and qPCR protocols previously optimized and tested for specificity were used for qPCR analyses of *P. irregulare*, *P. sylvaticum* Campbell and Hendrix, *P. ultimum* Trow, *P. vexans* de Bary and the genera *Cylindrocarpon* (four species) and *Phytophthora* (Tewoldemedhin et al., 2011a,b). The standard curves for these species were obtained using the following isolates, *P. irregulare* STE-U7193, *P. sylvaticum* STE-U7199, *P. vexans* STE-U6741 and *Phytophthora cactorum* STE-U7203, which were all isolated from ARD soil in a previous study and were identified using sequence analyses (Tewoldemedhin et al., 2011b). The *P. ultimum* isolate PPRI 8615 was isolated from grapevine roots (Spies, 2010). Isolates with “STE-U” codes have been deposited to the Stellenbosch University culture collection and the PPRI 8615 isolate is available from the culture collection of the Plant Protection Research Institute (Agriculture Research Council, Plant Protection Research Institute, South Africa). *Pythium ultimum* and *P. vexans* DNA was quantified using primer pairs PulF2 and PulR2, and PvF1 and PvR1 respectively that target the ITS region, using TaqMan technology (Spies, 2010). *Pythium irregulare*, *P. sylvaticum* and the genus *Phytophthora* were quantified using primer pairs PirF1 and PirR2 (Spies, 2010), Syl1F and Syl2R (Schroeder et al., 2006) and Yph1 and Yph2 (Schena et al., 2008) with SyberGreen I technology (Tewoldemedhin et al., 2011b). The *P. irregulare* and *P. sylvaticum* primers both target the ITS region, whereas the *Phytophthora* genus primers targets the ras-related protein (*Ypt1*) gene. The PCR products of a representative sample of orchard roots that tested positive for *Phytophthora* was sequenced to determine species identity. *Cylindrocarpon* was quantified using primer pair YTF2 and Cyl-R, which targets the ITS region of four apple associated *Cylindrocarpon* species including *C. destructans*, *C. liriodendri*, *C. pauciseptatum* and *C. macrodidymum* (Tewoldemedhin et al., 2011a).

qPCR analyses of *R. solani* AG-5 were conducted using primers RSAG5F (5'-GATATTTGGTTGTAGCTGGCTCATG-3') and RSAG5R (5'-GCACCAATTGTTCTTAAAAACAATC-3') (Mazzola and Zhao, 2010), and pure culture DNA of *R. solani* AG-5 isolate 5–104 obtained from ARD soil in Washington State, USA. The specificity of the qPCR reactions was tested using binucleate *Rhizoctonia* isolates associated with ARD in South Africa (Tewoldemedhin et al., 2011b). The final optimized conditions for amplification of *R. solani* AG-5 consisted of a total reaction volume of 40  $\mu\text{L}$  containing 4  $\mu\text{L}$  pure culture DNA extract (10 ng/ $\mu\text{L}$ ), 20  $\mu\text{L}$  SensiMix, 1  $\mu\text{L}$  of SYBR® Green I and 0.3  $\mu\text{M}$  of primer RSAG5F and 0.9  $\mu\text{M}$  of primer RSAG5R. Amplification was conducted using cycle conditions of initial denaturing at  $95^\circ\text{C}$  for 10 min followed by 40 cycles of denaturing at  $95^\circ\text{C}$  for 10 s, annealing at  $60^\circ\text{C}$  for 15 s and extension at  $72^\circ\text{C}$  for 30 s.

### 2.5.1. Correlation between pathogen DNA concentrations in roots with and seedling growth

Statistical analyses were conducted using the fungal and oomycete root DNA concentration data, as determined using qPCR, and seedling growth (reduction in weight and height) data obtained from the six non-treated orchard soils. Pearson's product moment correlation test was performed to determine the correlation between the two measured parameters (Otto, 1998).

### 2.6. Pathogenicity testing of actinomycetes in single inoculations or co-inoculations with *P. irregulare* or *C. macrodidymum*

#### 2.6.1. Actinomycete inoculum preparation

Thirty seven actinomycete isolates, representing the different actinomycete genera and genotypes isolated from seedling roots grown in non-treated and pasteurized soil, were assessed for pathogenicity toward apple. Actinomycete inoculum was prepared by mixing actinomycete spores with talc powder. Six grams of talc powder (for each actinomycete isolate) was first autoclaved for 20 min, and actinomycete spores obtained from 2-week-old YEME agar plates were mixed with the talc powder. This mixture was used to inoculate the plant growth medium, after which actinomycete densities were determined per gram of planting medium, one day after inoculation (see below in Section 2.6.3).

#### 2.6.2. *P. irregulare* and *C. macrodidymum* inoculum preparation

One isolate each of *P. irregulare* (STE-U7193) and *C. macrodidymum* (STE-U6598) that were previously isolated from apple roots, and which were shown to be highly virulent when inoculated individually (Tewoldemedhin et al., 2011a,b), were included in the pathogenicity trials. The isolates were identified using sequence analyses (GenBank accessions JF690897 and GU183634), and can be obtained from the Stellenbosch University culture collection. Inoculum of *Cylindrocarpon* was prepared using millet seed as previously described (Strauss and Labuschagne, 1995; Tewoldemedhin et al., 2011a). *Pythium irregulare* inoculum was prepared using sand-bran as previously described (Lamprecht, 1986; Tewoldemedhin et al., 2011b).

#### 2.6.3. Inoculation of planting medium and planting of glasshouse pathogenicity trial

Two experimental protocols were conducted, each repeated once. In the first, the pathogenicity of 37 actinomycete isolates was determined, and in the second the pathogenicity of 11 actinomycete isolates in co-inoculation with *P. irregulare* or *C. macrodidymum* was assessed. The pathogenicity of *P. irregulare* and *C. macrodidymum* when co-inoculated was also investigated. The 11 actinomycete isolates were selected to represent the different genotypes identified within the genus *Streptomyces* and the different orchard sources of these isolates. The treatments used in the co-inoculation trial are shown in Table 7.

The planting medium consisted of a 2:1 (v/v) ratio of bark medium and sand, and the pH was corrected to 6.5 by adding dolomitic lime. Three days prior to inoculation, the growth medium was pasteurized as described above. Planting medium was inoculated with an individual actinomycete isolate by mixing approximately 6 g of the talc inoculum with 6 L of pasteurized planting medium that were incubated overnight under ambient conditions. The subsequent day, actinomycete densities were determined through serial dilution plating onto CS medium, and adjusted to  $1\text{--}5 \times 10^7$  cfu g<sup>-1</sup> dry planting medium. The control consisted of planting medium mixed with talc powder. Planting medium was inoculated with *C. macrodidymum* or *P. irregulare* by adding sand-bran and millet seed inoculum, respectively, at a rate of 1% v/v each. The *Pythium* and *Cylindrocarpon* inoculated plant growth

media were incubated for 24 h at 20–25 °C prior to planting. In co-inoculation experiments, actinomycete inoculum densities were equivalent to that used when isolates were inoculated in single culture. The control for co-inoculation studies consisted of plant growth medium inoculated with both sand-bran and millet seed, each at a rate of 1% v/v. Four-week-old apple (Golden Delicious) seedlings were used in the pathogenicity trials. The experimental design and growth conditions of the trial were as described in Section 2.2, except that only five replicates were used for each treatment.

#### 2.6.4. Evaluation of pathogenicity trial

Seedling growth (increase in weight and height) was assessed after three months, as described in Section 2.2. In order to fulfill Koch's postulate, isolations for actinomycetes, *Cylindrocarpon* and *Pythium* were made from fibrous roots of seedlings grown in specific treatments inoculated with these microbes, as well as the un-inoculated controls. Isolations for actinomycetes were made as described above, and for *C. macrodidymum* and *P. irregulare* as previously described (Tewoldemedhin et al., 2011a,b).

DNA concentrations of *P. irregulare* and *Cylindrocarpon* spp. in seedling roots from treatments that were inoculated or co-inoculated with these pathogens were also determined using qPCR analyses. qPCR analyses for these pathogens were conducted as described in Section 2.5. Statistical analyses of the correlation between *Cylindrocarpon* and *Pythium irregulare* DNA concentrations in roots and weight and height of seedlings were conducted as described in Section 2.5.1.

### 2.7. Statistical analysis

Statistical analyses were conducted on seedling weight and height data. Levene's variance ratio test (Levene, 1960) was performed to test for homogeneity of trial variances between the trial repeats. Data of the two independent trials were considered block treatments providing that Levene's variance ratio test showed homogeneity in trial variance. Data were also subjected to analysis of variance (SAS, 1999), and the Shapiro–Wilk test was performed to test for normality (Shapiro and Wilk, 1965). In cases where deviations from normality were due to kurtosis and not skewness, the data were accepted as reliable and the results were interpreted without transformation (Glass et al., 1972). The student's *t*-Least Significant Difference was calculated at the 5% confidence level to compare treatment means.

## 3. Results

### 3.1. Soil analyses

Characteristics of the six soils are presented in Table 1. Three of the orchards had a loam soil texture, two had a sandy texture and the other had a clay texture. The pH of the sampled soils ranged from pH 5.5 to 6.6. The two orchards (O2 and O6) that had the most severe ARD, have a sandy soil texture as well as high phosphorus content (Table 1).

### 3.2. Biocide and replant growth inhibition trials

The error variance ratios for the apple seedling weight and height were  $P = 0.314$  and  $P = 0.756$  respectively, based on Levene's variance ratio test (Levene, 1960). Consequently, data from the two trials could be combined in all the analyses. Analyses of variance on seedling weight and height showed that there were significant treatment  $\times$  orchard interactions for weight and seedling height (Table 2).

**Table 2**

Analysis of variance for the effect of six treatments on mean weight and height of apple seedlings grown in six apple replant orchard soils for three months under glasshouse conditions.

Source of variation	DF <sup>a</sup>	Weight		Height	
		MS <sup>b</sup>	SL <sup>c</sup>	MS <sup>b</sup>	SL <sup>c</sup>
Trial	1	515.70	0.002	221.45	0.003
Blocks (Trial)	10	62.66	0.321	25.34	0.440
Orchard	5	2311.41	<0.0001	458.52	<0.0001
Treatment	5	1765.90	<0.0001	703.37	<0.0001
Treatment × Orchard	25	126.37	0.0004	51.66	0.003
Error	385	54.28		25.27	
Corrected Total	431				

<sup>a</sup> DF = degree of freedom.

<sup>b</sup> MS = mean square.

<sup>c</sup> SL = Significance level.

The severity of ARD within each of the six soils was calculated as the growth (weight) response in the pasteurized soil of each orchard soil, relative to the growth (weight) in the non-treated soil of the corresponding orchard. The soils could be classified into three significantly different severity groups, i.e. low (Orchards O1 and O4), medium (Orchards O3 and O5) and severe (Orchards O2 and O6). As expected, the height data was less informative for grouping of the orchards soils into severity levels (data not shown), since in previous pathogenicity studies (Tewoldemedhin et al., 2011a,b) the largest effect of replant pathogens was seen on plant weight, and significant differences were only occasionally seen with plant height (Table 1). This relative rating of ARD severity was supported by diluting 15% of each orchard soil into pasteurized soil of the corresponding orchard, which showed that dilution of soil from the two orchards with low ARD severity (O1 and O4) did not result in a significant difference in the height or weight of these seedlings when compared to seedlings grown in the corresponding pasteurized orchard soil (Table 3). On the other hand, seedlings grown in similarly diluted soil from orchards with moderate (O3 and O5) and severe (O2 and O6) ARD still exhibited significant reductions in weight and height compared to the pasteurized control (Table 3).

Treatment of orchard soils with difenconazole to suppress several fungi including *Cylindrocarpon*, *Fusarium* and *Rhizoctonia* only resulted in a significant increase in seedling growth in O3 orchard soil. This growth response was, however, still significantly less than that attained in response to pasteurization of this orchard soil (Table 3).

Metalaxyl application significantly increased seedling weight or height in four of the six orchard soils (Table 3) but did not alter seedling growth in soils from the O4 and O6 orchards (Table 3). In three of the orchard soils, O1, O4 and O5, metalaxyl application was

able to improve seedling growth to the level obtained in the corresponding pasteurized soil of each orchard. Thus, in Orchard O4, although metalaxyl did not significantly improve seedling growth (weight and height) relative to the non-treated control, it was able to restore seedling health to a status that was comparable to that attained in pasteurized soil (Table 3).

Treatment of soil with fenamiphos to suppress nematodes resulted in a significant growth response (either weight or height) in two of the six orchards. In soils collected from Orchards O4 and O6, fenamiphos treatment resulted in a significant improvement in the height and weight of apple seedlings respectively, compared to seedlings in the untreated control soils. Orchard O4 was the only orchard where the increase in seedling growth (weight and height) resulting from fenamiphos application was equivalent to that obtained in response to soil pasteurization (Table 3).

### 3.3. Isolation and identification of actinomycete genera and genotypes

The sterilization method and plating media most likely affected the relative recovery of actinomycetes from apple roots. Seventy six percent of the isolates were recovered from the group A roots, whereas only 24% of the isolates were recovered from group B roots. This could be due to the superior elimination of bacteria other than actinomycetes through triple sterilization of group A roots, but more likely is due to selective nature of the CS media onto which group A roots were plated relative to the water agar onto which group B roots were plated.

For each of the orchards a comparable number of actinomycetes were obtained from the pasteurized and non-treated soils, but in general the numbers obtained were low (Table 4). Considering the total number of roots plated from both experiments, actinomycetes were recovered from 0.49% to 0.97% of roots from seedlings grown in non-treated soil, and 0.07%–0.76% of roots from the pasteurized soil treatment.

In total, ninety six actinomycete isolates were recovered from the six ARD soils. Sequence analyses of the 16S rRNA gene showed that 95.83 % of the isolates belonged to the genus *Streptomyces*, whereas 4.17% belonged to the genus *Nocardiopsis* (Table 4). The identification of *Streptomyces* species is difficult because substantial controversy exists as to which species are valid, and the means by which species are delimited. Furthermore, for several of the BLAST hits the similarity to GenBank *Streptomyces* sequences of known species was low (less than 98%) or to an unknown *Streptomyces* species. Therefore, *Streptomyces* species were not identified, but were grouped into five Genotypes. Based on the sequence similarity most of the isolates (58.33%) were grouped into one large group designated as Genotype 5, all having similarity to an unknown

**Table 3**

Mean seedling weight and height of apple seedlings in response to six treatments applied to six different apple replant orchard soils.

Treat <sup>a</sup>	Weight <sup>b</sup>						Height <sup>b</sup>					
	O1	O2	O3	O4	O5	O6	O1	O2	O3	O4	O5	O6
P	40.8bcd <sup>c</sup>	45.2ab	32.5e–h	32.3e–h	41.6bc	49.4a	21.5abc	23.8ab	17.4d–g	17.8c–g	24.8a	24.9a
PN	40.6bcd	37.2cde	20.4mno	29.8g–k	30.0g–k	30.6g–k	21.3a–d	19.8c–f	10.1no	14.9g–l	15.8f–j	17.2e–h
D	34.4efg	30.8f–k	17.3no	26.0j–m	25.4j–m	32.4e–h	15.2g–k	14.5g–l	11.1lmn	12.5j–n	11.4k–n	15.3g–k
M	40.6bcd	35.3d–g	22.7lmn	27.9h–l	36.6c–f	31.2f–j	17.6c–g	16.1f–j	13.2h–n	14.6g–l	17.2e–h	16.7e–i
F	32.8e–h	32.1e–i	18.4no	33.9efg	30.9f–k	34.6efg	14.6g–l	15.8f–j	10.3mno	18.1c–g	16.2f–j	20.5b–e
NT	32.5e–h	26.3i–m	16.1o	25.0klm	26.1j–m	30.8f–k	16.7e–i	12.6i–n	6.5o	15.8f–j	13.0i–n	14.4g–m
LSD <sub>0.05</sub>	5.9						4.04					

<sup>a</sup> Pasteurized orchard soil (P), 15% non-treated orchard soil added to pasteurized orchard soil (PN), metalaxyl (M), difenconazole (D) and fenamiphos (F) treated orchard soil and non-treated orchard soil (NT).

<sup>b</sup> Values are the mean seedling weight (g) and height (cm) increase of apple seedlings that were grown for three months in the soil. The values are the means of two independent experiments that each contained six replicates.

<sup>c</sup> Means within each parameter followed by the same letter do not differ significantly ( $P = 0.05$ ).

**Table 4**

Genera of actinomycetes and genotypes within the genus *Streptomyces* recovered from the roots of three-month-old apple seedlings that were grown in steam pasteurized and non-treated soils collected from six apple replant disease orchards.

Actinomycete genus and genotypes within the genus <i>Streptomyces</i>	Orchard											
	O1		O2		O3		O4		O5		O6	
	P <sup>a</sup>	NT <sup>b</sup>	P	NT	P	NT	P	NT	P	NT	P	NT
<i>Streptomyces</i> genotype 1 <sup>c</sup>	2	5	3	7	4	9	3	3	2	11	–	7
<i>Streptomyces</i> genotype 2	3	1	1	–	1	2	1	1	–	2	1	1
<i>Streptomyces</i> genotype 3	1	1	–	1	4	2	–	–	–	1	–	–
<i>Streptomyces</i> genotype 4	2	–	–	–	2	–	–	1	4	–	–	–
<i>Streptomyces</i> genotype 5	1	–	–	–	–	–	–	1	–	–	–	1
<i>Nocardioopsis</i> sp.	–	1	–	2	–	–	–	1	–	–	–	–
Total	9	8	4	10	11	13	4	7	6	14	1	9

<sup>a</sup> Soils were steam pasteurized for three hours on two consecutive days.

<sup>b</sup> Soils were left un-treated.

<sup>c</sup> Sequences that are representative of the five *Streptomyces* genotypes were submitted to GenBank. Genotype 1 is represented by accessions JF690898 and JF690899, Genotype 2 by JF690900 and JF690901, Genotype 3 by JF690902 and JF690903, Genotype 4 by JF690904 and JF690905, Genotype 5 by JF690906, JF690907 and JF690908, and *Nocardioopsis* sp. by JF690909 and JF690910.

*Streptomyces* species. The remaining isolates were designated as Genotype 1 (10.42% of isolates) that had highest similarity to *S. anulatus*, Genotype 2 (14.58% of isolates) that had highest similarity to *S. canus*, Genotype 3 (9.38% of isolates) that had highest similarity to *S. lividans* and Genotype 4 (3.12% of isolates) that had highest similarity to *S. vinaceus* (Table 4).

### 3.4. Nematode isolation and identifications

The number of nematodes in each of the orchards was dominated by saprophytes, whereas parasitic nematode genera were only detected in some orchards. Orchard O5 was the only orchard that contained high numbers of plant parasitic nematodes, specifically *Pratylenchus penetrans* (Table 5). Orchards O1, and O4 contained very low levels of *Pratylenchus*, which belonged to species other than *P. penetrans*, i.e. *P. scribneri* in orchard O1 and *P. delattrei* in Orchard O4. In Orchard O6, several different nematode genera (*Pratylenchus*, *Xiphinema* and *Paratrichodorus*) were found that are considered as harmful toward apple, but these were present at low levels (Table 5). The specific species of the genera in this orchard were *Pratylenchus scribneri*, *Xiphinema taylora* and *Paratrichodorus porosus*.

### 3.5. Quantitative real-time PCR analyses of replant fungal and oomycete pathogens

qPCR analyses showed that *Cylindrocarpum* was present in all six orchards at varying DNA concentrations (Table 6). There was no

significant negative correlation between the *Cylindrocarpum* DNA concentrations in seedling roots, and the growth (weight,  $P = 0.592$  and height,  $P = 0.660$ ) of seedlings.

*Phytophthora* DNA was detected in all six orchards, with the highest DNA concentration present in Orchard O5 that had moderately severe ARD (Table 6). Sequence analyses of the qPCR products revealed that only *P. cactorum* was present in the samples, since only one set of chromatograph peaks were observed in all the chromatograms. There was no significant negative correlation between *Phytophthora* DNA concentrations in seedling roots, and the growth (weight,  $P = 0.220$  and height,  $P = 0.292$ ) of seedlings.

The highest *P. irregulare* DNA concentration was detected in roots of seedlings grown in O4 orchard soil, which had low ARD severity. Seedlings grown in other orchard soils all yielded low *P. irregulare* DNA concentrations ranging from 2 to 8 fg  $\mu\text{L}^{-1}$  root DNA (Table 6). There was no significant negative correlation between *P. irregulare* DNA concentration in roots and the growth (weight,  $P = 0.285$  and height,  $P = 0.435$ ) of seedlings.

*Pythium sylvaticum* DNA was only detected in roots of seedlings grown in soil from the O3 orchard that had moderate ARD severity (Table 6). There was a significant negative correlation between *P. sylvaticum* DNA concentrations in the roots of seedlings grown in the O3 orchard soil and seedling weight ( $r = -0.473$ ;  $P = 0.002$ ) and height ( $r = -0.471$ ;  $P = 0.002$ ).

Of all the pathogens investigated, the highest DNA concentrations were obtained for *P. vexans*. Standard deviations obtained for *P. vexans* DNA concentrations were also the largest (Table 6), which were caused by a diverse range of DNA concentrations obtained for

**Table 5**

The average number of nematodes obtained from the roots and soils of apple seedlings grown in six apple replant disease orchard soils.

Genus	O1 <sup>a</sup>		O2		O3		O4		O5		O6	
	Root <sup>b</sup>	Soil <sup>c</sup>	Root	Soil	Root	Soil	Root	Soil	Root	Soil	Root	Soil
Saprophytes	118	100	180	202	13	96	154	204	207	168	384	94
<i>Pratylenchus</i>	1	0	0	0	0	0	8	8	104	20	12	0
<i>Meloidogyne</i>	0	0	0	0	0	0	0	0	0	0	0	0
<i>Criconeumatinae</i>	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spirals</i>	3	6	2	0	0	0	0	0	9	0	0	0
<i>Xiphinema</i>	0	0	0	0	0	0	0	0	0	0	0	32
<i>Paratrichodorus</i>	0	0	0	0	0	0	0	0	0	0	11	0
<i>Hemicycliphora</i>	0	0	0	0	4	0	0	0	0	0	0	0

<sup>a</sup> Six apple orchards were investigated including orchards GG, GE, GM, CV, CD and CE.

<sup>b</sup> The number of nematodes is the average of two repeat glasshouse trials after seedlings were grown for 3 months in the soils. The nematode numbers are per 1 g of root material.

<sup>c</sup> The number of nematodes is the average of two repeat glasshouse trials after seedlings were grown for 3 months in the soils. The nematode numbers are per 100 cm<sup>3</sup> of soil sample.

**Table 6**  
Mean DNA concentrations of pathogenic fungal and oomycete genera and species in apple seedling roots that were grown in six apple replant disease orchard soils, as assessed through quantitative real-time PCR analyses.

Pathogen	Mean pathogen DNA concentration (fg $\mu\text{L}^{-1}$ ) $\pm$ standard deviation <sup>a</sup>					
	O1 (low)	O2 (moderate)	O3 (severe)	O4 (low)	O5 (moderate)	O6 (severe)
<i>Cylindrocarpon</i> (minimum) <sup>b</sup>	1103 $\pm$ 317	2886 $\pm$ 2002	3742 $\pm$ 2621	2561 $\pm$ 679	802 $\pm$ 839	5988 $\pm$ 6098
<i>Cylindrocarpon</i> (maximum) <sup>b</sup>	3942 $\pm$ 1105	9716 $\pm$ 6324	12,759 $\pm$ 8609	8948 $\pm$ 2263	2911 $\pm$ 2911	19,821 $\pm$ 19,179
<i>Cylindrocarpon</i> (mean) <sup>c</sup>	2523 $\pm$ 711	6301 $\pm$ 4163	8251 $\pm$ 5615	5755 $\pm$ 1471	1857 $\pm$ 1875	12,905 $\pm$ 12,639
<i>Phytophthora</i> spp.	1968 $\pm$ 967	4275 $\pm$ 4344	1536 $\pm$ 1914	26.83 $\pm$ 65	14806 $\pm$ 11399	8574 $\pm$ 10,278
<i>P. irregulare</i>	7 $\pm$ 8	5 $\pm$ 4	3 $\pm$ 5	34 $\pm$ 32	8 $\pm$ 6	2 $\pm$ 2
<i>P. sylvaticum</i>	0	0	12 $\pm$ 10	0	0	0
<i>P. vexans</i>	42,333 $\pm$ 54,325	75,988 $\pm$ 77,648	50,427 $\pm$ 80,513	136,737 $\pm$ 2,469,490	12,310 $\pm$ 206,230	118,555 $\pm$ 2,633,510
<i>P. ultimum</i>	9 $\pm$ 10	10 $\pm$ 19	2 $\pm$ 3	3 $\pm$ 3	2 $\pm$ 4	2 $\pm$ 3
<i>R. solani</i> AG-5	0	0	0	0	0	0
Total pathogen DNA	51,885 $\pm$ 57,443	99,181 $\pm$ 94,494	76,732 $\pm$ 99,290	154,964 $\pm$ 2,474,003	32,696 $\pm$ 223,264	165,847 $\pm$ 2,681,709

<sup>a</sup> Mean DNA concentrations were determined in apple seedling roots that were grown in soil obtained from six apple orchards (O1–O6). The severity of apple replant disease in each orchard is shown in brackets following the orchard names. Mean DNA concentration for each orchard was determined from seedling roots grown in six soil replicates of each orchard. The data is from one trial.

<sup>b</sup> Amplification with the *Cylindrocarpon* primers that simultaneously detect four apple associated *Cylindrocarpon* species, yields different standard curves for each of the species. Therefore, the mean *Cylindrocarpon* DNA concentrations were quantified using standard curves of the two *Cylindrocarpon* species that yields the lowest (*C. liriodendri*) and highest (*C. destructans*) amount of DNA.

<sup>c</sup> Average of the minimum and maximum *Cylindrocarpon* concentrations that were determined using the standard curves of *C. destructans* and *C. liriodendri* respectively.

each of the six replicates within the glasshouse trial for some orchards (O4, O5 and O6). Since *P. vexans* was not previously detected from any of the six orchards by isolation (Tewoldemedhin et al., 2011b), identity of the amplicons from different orchards were sequenced, which confirmed that the PCR products were *P. vexans*. There was no significant negative correlation between the amount of *P. vexans* DNA in seedling roots and the growth (weight,  $P=0.412$  and height,  $P=0.162$ ) of seedlings.

Similar to *P. vexans*, *P. ultimum* DNA was detected in the roots of seedlings planted in all orchard soils. The identity of the PCR products generated using *P. ultimum*-specific primers was confirmed through sequence analyses, since this species was not previously identified through isolation studies in the six orchards (Tewoldemedhin et al., 2011b). The DNA concentrations of *P. ultimum* detected in seedling roots were among the lowest of the different pathogens. There was no significant negative correlation between *P. ultimum* DNA concentrations in seedling roots and the growth (weight  $P=0.924$  and height  $P=0.886$ ) of seedlings.

The standard curve of the *R. solani* AG-5 qPCR analysis showed that the lowest amount of DNA that could be detected from pure culture DNA was 10 fg  $\mu\text{L}^{-1}$ . None of the six orchard soils tested positive for *R. solani* AG-5 DNA.

### 3.6. Pathogenicity of actinomycetes

In pathogenicity assays conducted with individual actinomycete isolates, the error variance ratios between the two trials for apple seedling height and weight were  $P=0.933$  and  $P=0.002$  respectively. Therefore, weighted analysis was conducted for weight in order to combine the data of the two trials (John and Quenouille, 1977). In the experiments where the pathogenicity of 11 *Streptomyces* isolates was assessed in co-inoculations with *C. macrodidymum* or *P. irregulare*, the error variance ratios between trials for increase in seedling weight and height were  $P<0.001$  and  $P=0.037$  respectively, and therefore weighted analysis was also done in order to combine the two data sets.

In single isolate pathogenicity assays, the analyses of variance on the actual mean weight and height of apple seedlings did not show a significant interaction for treatment and trial ( $P=0.874$  for weight, and  $P=0.983$  for height), and treatment also did not significantly affect weight ( $P=0.304$ ) and height ( $P=0.928$ ). Thus, none of the actinomycete isolates were able to significantly affect seedling height or weight. Inoculation of the growth media with

actinomycetes was considered successful since actinomycetes were re-isolated from the surface sterilized roots of the inoculated treatments, but not from the control treatment containing only talc powder.

Analyses of variances for the co-inoculation experiments involving *Streptomyces* isolates with *P. irregulare* or *C. macrodidymum* showed that treatment significantly affected seedling weight ( $P<0.001$ ) and height ( $P<0.001$ ) in both trials. For seedling weight, there was also a significant treatment and trial interaction between the two trials ( $P=0.002$ ). Similar to the first experiment, in general the individual inoculated *Streptomyces* isolates did not cause a significant reduction in seedling weight or height when compared to any of the three inoculated controls. The only exception to this were individual inoculations of the two *Streptomyces* genotype 2 isolates (S. genotype 2.1 and S. genotype 2.2) that resulted in a significant reduction of seedling height, although marginal, when compared to control 1 (sand-bran and talc media) and control 2 (sand-bran and millet seed) but not control 3 (millet seed and talc medium). In general, co-inoculation of *Streptomyces* isolates with the known pathogens *P. irregulare* or *C. macrodidymum* did not significantly alter plant growth relative to that attained in the presence of these pathogens alone (Table 7). The sole exception to this trend was the significant, though marginal, increase in height observed for seedlings grown in soils co-inoculated with *P. irregulare* and the S. genotype 2.1 isolate (Table 7).

Individual and co-inoculation of the *P. irregulare* and *C. macrodidymum* isolates resulted in significant seedling growth reductions. Co-inoculation of *P. irregulare* and *C. macrodidymum* caused a significant reduction in seedling weight compared to treatments that only contained one of the pathogens. Co-inoculation of *C. macrodidymum* with *P. irregulare* also resulted in a significant reduction in seedling height compared to that of seedlings grown in medium inoculated with *C. macrodidymum* alone (Table 7).

qPCR analyses of *P. irregulare* and *C. macrodidymum* in seedling root DNA showed that the concentration of each pathogen was similar whether the isolate was co-inoculated or whether it was present individually. *C. macrodidymum* inoculated seedlings had a mean DNA concentration of 10,830  $\pm$  4219 (fg  $\mu\text{L}^{-1}$ ), whereas when this pathogen was co-inoculated with *P. irregulare* it was detected at a DNA concentration of 12,015  $\pm$  2455 fg  $\mu\text{L}^{-1}$ . *Pythium irregulare* had a DNA concentration of 1  $\pm$  0.8 fg  $\mu\text{L}^{-1}$  in seedling roots when inoculated as a single culture, whereas co-inoculation of the pathogen with *C. macrodidymum* resulted in a DNA

**Table 7**

Effect of co-inoculation of 11 *Streptomyces* isolates with *Pythium irregulare* and *Cylindrocarpon macrodidymum* on apple seedling weight and height.

Treatment	Weight <sup>a</sup>	Height <sup>a</sup>
Control1 <sup>b</sup>	31.6ab	17.0ab
Control2	33.2a	17.5a
Control3	31.8ab	16.2a–d
<i>Pythium irregulare</i>	21.9d	11.4l
<i>Cylindrocarpon macrodidymum</i>	26.1c	13.5gh
<i>P. irregulare</i> + <i>C. macrodidymum</i>	19.8e	10.6l
<i>Streptomyces</i> genotype 1.1	32.4ab	16.4a–d
<i>Streptomyces</i> genotype 1.2	31.9ab	16.6a–d
<i>Streptomyces</i> genotype 2.1	32.1ab	15.1def
<i>Streptomyces</i> genotype 2.2	32.2ab	15.4cde
<i>Streptomyces</i> genotype 3.1	31.0b	16.9ab
<i>Streptomyces</i> genotype 3.2	31.8ab	16.1a–d
<i>Streptomyces</i> genotype 4.1	31.2b	16.5a–d
<i>Streptomyces</i> genotype 4.2	32.0ab	15.9bcd
<i>Streptomyces</i> genotype 5.1	31.6ab	16.9abc
<i>Streptomyces</i> genotype 5.2	31.9ab	16.1a–d
<i>Streptomyces</i> genotype 5.3	32.3ab	17.3ab
<i>C. macrodidymum</i> + <i>S.</i> genotype 1.1	25.8c	13.8fg
<i>C. macrodidymum</i> + <i>S.</i> genotype 1.2	26.8c	13.6g
<i>C. macrodidymum</i> + <i>S.</i> genotype 2.1	25.8c	13.9efg
<i>C. macrodidymum</i> + <i>S.</i> genotype 2.2	26.0c	13.2gh
<i>C. macrodidymum</i> + <i>S.</i> genotype 3.1	25.7c	13.1g–j
<i>C. macrodidymum</i> + <i>S.</i> genotype 3.2	26.6c	13.5gh
<i>C. macrodidymum</i> + <i>S.</i> genotype 4.1	26.5c	13.3gh
<i>C. macrodidymum</i> + <i>S.</i> genotype 4.2	25.4c	13.2g–j
<i>C. macrodidymum</i> + <i>S.</i> genotype 5.1	26.2c	13.7fg
<i>C. macrodidymum</i> + <i>S.</i> genotype 5.2	26.5c	13.5gh
<i>C. macrodidymum</i> + <i>S.</i> genotype 5.3	26.1c	13.6g
<i>P. irregulare</i> + <i>S.</i> genotype 1.1	22.0d	11.3l
<i>P. irregulare</i> + <i>S.</i> genotype 1.2	21.9d	11.2l
<i>P. irregulare</i> + <i>S.</i> genotype 2.1	21.9d	12.9g–k
<i>P. irregulare</i> + <i>S.</i> genotype 2.2	20.8d	11.7i–l
<i>P. irregulare</i> + <i>S.</i> genotype 3.1	22.2d	11.1l
<i>P. irregulare</i> + <i>S.</i> genotype 3.2	21.2d	11.0l
<i>P. irregulare</i> + <i>S.</i> genotype 4.1	22.0d	11.8i–l
<i>P. irregulare</i> + <i>S.</i> genotype 4.2	22.0d	11.7jkl
<i>P. irregulare</i> + <i>S.</i> genotype 5.1	22.2d	11.6kl
<i>P. irregulare</i> + <i>S.</i> genotype 5.2	20.9d	12.0h–l
<i>P. irregulare</i> + <i>S.</i> genotype 5.3	21.2d	11.4l
LSD <sub>0.05</sub>	1.7	1.5

<sup>a</sup> Mean seedling weight (g) and height (cm) increase, three months after trial inoculation. The data is the average of two independent experiments that each contained five replicates for each treatment.

<sup>b</sup> The media that were used to inoculate the *Streptomyces* (talc powder), *Pythium irregulare* (sand bran) and *Cylindrocarpon macrodidymum* (millet seed) were different. Therefore, three different controls were included that represented the different combinations of inoculation media, i.e. control 1 is the control used for sand-bran medium and talc medium inoculated together, control 2 is the control used for sand-bran medium and millet seed inoculated together and control 3 is the control used for millet seed inoculum and talc medium inoculated together.

concentration of  $1 \pm 0.7 \text{ fg } \mu\text{L}^{-1}$ . There was a significant negative correlation between the *P. irregulare* DNA concentrations and the increase in seedling weight ( $r = -0.544$ ;  $P < 0.001$ ) and height ( $r = -0.635$ ;  $P < 0.001$ ).

All introduced organisms were re-isolated from the inoculated plant material in the combined pathogenicity trials, but not from the control plants. All isolates obtained had characteristics similar to the original cultures.

## 4. Discussion

### 4.1. Biocide application and qPCR reveal the importance of multiple biological agents

Our study showed that six South African apple orchard soils that were previously cultivated to apple for more than 15 years all had some degree of ARD. Two of the soils (Orchards O1 and O4) had low

ARD severity, two soils (Orchards O3 and O5) had moderately severe ARD and two of the soils (Orchards O2 and O6) had severe ARD based on the growth response (weight not height) of apple seedlings in pasteurized versus non-treated soils. This classification of the soils and the biological nature of the problem were also supported by the fact that the disease status of the soils could be re-established in pasteurized soil by introduction of 15% of the native moderately and severe ARD soils. Based on qPCR analyses several of the ARD 'marker' microbes including *P. irregulare*, *P. vexans*, *P. ultimum* and the genera *Phytophthora* and *Cylindrocarpon* were present in most of the test orchard soils, whereas *P. sylvaticum* was less frequently encountered. The importance of *Pythium* and *Phytophthora* in ARD was further highlighted by the significant increase in seedling growth response in four of the orchards following the application of metalaxyl, a biocide with specific activity against these pathogens. In contrast, plant parasitic nematodes were rarely detected at densities sufficient to impair plant growth (Barker and Olthof, 1976). This was also supported by the application of fenamiphos that only positively affected seedling growth in two of the orchards. This suggests that the biological agents and interactions between agents are complex and vary in the soils.

### 4.2. Synergistic interaction between *Cylindrocarpon* and *Pythium* contribute to ARD, but not actinomycetes

Support for the complex interactions in ARD was shown through the synergistic disease causing effect of *P. irregulare* and *C. macrodidymum* when co-inoculated onto seedlings. Braun (1991, 1995) made a similar observation, and found that growth suppression of apple was greater in co-inoculations of *Pythium* spp. and *Cylindrocarpon lucidum* than when either of these pathogens were present individually. Culturable actinomycetes are not likely to have a significant role as causal agents of ARD in South Africa. These bacteria were incapable of reducing apple seedling growth on their own or exacerbating plant damage in concert with the known pathogens *P. irregulare* or *C. macrodidymum*.

### 4.3. Pathogen DNA concentration in roots is not correlated with seedling growth inhibition

In general there were no significant negative correlations between DNA concentrations of the ARD marker microbes in roots and seedling growth inhibition. Furthermore, specific pathogen DNA concentrations were not notably higher in seedling roots grown in severe and moderately severe ARD soils, than in low ARD severity orchards. This could be explained by several factors. First of all, pathogens attack different sections of the seedling root systems (Agrios, 2005). Therefore, the very low root DNA concentrations of *P. ultimum*, *P. sylvaticum* and *P. irregulare* may be due to these pathogens mainly functioning as root pruners (Martin and Loper, 1999). In contrast, root DNA concentrations of *P. vexans* and *Phytophthora* were detected at much higher concentrations, suggesting that these pathogens may be more prone to colonize larger roots instead of being fine-root pruners. This is an interesting observation in view of the fact that *P. vexans* has been hypothesized as being more closely related to *Phytophthora* than to *Pythium*. Therefore, it has been suggested recently that *Pythium* species in clade K, into which *P. vexans* falls, should be placed into a new genus named *Phytophythium* (Bala et al., 2010; Lévesque and De Cock, 2004). Secondly, the pathogens may differ in the mechanisms by which they invade roots and cause host damage. Some isolates may have a higher potential for causing host cell death through the production of cell wall enzymes, toxins or effectors, and thus do not have to establish extensive host colonization in order to reduce host growth. For example, some soil-borne pathogens, including

*C. didymum*, produce mycotoxins that cause stunting of plant root systems and thus plant growth (Barbetti, 2005), whereas the virulence of *Pythium* species has been attributed to their ability to produce cell-wall degrading enzymes and toxins (Van der Plaats-Niterink, 1981; Campion et al., 1997). Lastly, it could be that the synergistic interactions of pathogens in roots, rather than pathogen DNA concentrations as such, are more important in determining the severity of ARD. Some evidence for this was found in the *P. irregulare* and *C. macrodidymum* co-inoculation study where disease severity was significantly greater when the pathogens were co-introduced, but the DNA concentrations of *P. irregulare* were similar whether or not *C. macrodidymum* was present.

#### 4.4. Some aspects of ARD in South Africa differ from other regions

ARD in South African orchards, specifically in the Western Cape region, may differ in some aspects from ARD in other regions of the world where a drier climate exists. The concurrent occurrence of *P. ultimum* with at least two other highly pathogenic *Pythium* spp. (*P. irregulare* and *P. vexans*) within the same orchard, along with *Phytophthora*, for all orchards in South Africa was notable. Furthermore, *P. vexans* has only been identified as a pathogen of ARD in South Africa (Tewoldemedhin et al., 2011b). The prominence of highly virulent oomycetes, and the absence of *R. solani* AG-5, in South Africa could be due to the mild climate in winter and autumn, which is accompanied with high rainfall periods in this region.

#### 4.5. The multiphasic approach is important for elucidation ARD

Our multiphasic approach revealed important aspects that are relevant to ARD world-wide, and possibly soil sickness of other tree crops. The study re-emphasized the fact that elucidating the etiology of ARD is problematic because of the site-specificity of ARD, and interaction between various putative pathogens within specific sites. Nonetheless, some valuable conclusions can be made using a multiphasic approach. First of all, the use of biocide applications and the monitoring of marker microbes involved in ARD are important, since it proves the biological nature of the problem and the involvement of specific groups of pathogens. The ARD marker microbes that can be investigated in ARD soils include *P. irregulare*, *P. sylvaticum*, *P. vexans*, *P. ultimum*, *R. solani* AG-5 and the genera *Phytophthora* and *Cylindrocarpon*. Our study showed that actinomycetes are unlikely to be an ARD marker microbe. This should, however, also be investigated in other regions of the world, since limited information is available on this subject. Thirdly, investigations into nematodes in soil sickness are important since parasitic nematodes may cause damage at high or low population numbers by acting synergistically with fungal and oomycete pathogens in causing disease. Fourthly, qPCR analyses are very useful for high throughput and accurate detection of the marker microbes, but attempts to correlate the DNA concentrations of these microbes in seedling roots with ARD severity (seedling growth inhibition) do not yield useful information. The analyses of rhizosphere soil might be a better option for investigating if negative correlations exist between pathogen DNA concentrations and seedling growth inhibition. Lastly, our study highlighted the importance of synergistic interactions between ARD pathogens using artificial co-inoculation studies. Future studies should expand on these experiments in order to increase our understanding of interactions between ARD pathogens.

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